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Prolonged Exposure to Particulate Chromate Inhibits RAD51 Nuclear Import Mediator Proteins

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Abstract

Particulate hexavalent chromium (Cr(VI)) is a human lung carcinogen and a human health concern. The induction of structural chromosome instability is considered to be a driving mechanism of Cr(VI)-induced carcinogenesis. Homologous recombination repair protects against Cr(VI)-induced chromosome damage, due to its highly accurate repair of Cr(VI)-induced DNA double strand breaks. However, recent studies demonstrate Cr(VI) inhibits homologous recombination repair through the misregulation of RAD51. RAD51 is an essential protein in HR repair that facilitates the search for a homologous sequence. Recent studies show prolonged Cr(VI) exposure prevents proper RAD51 subcellular localization, causing it to accumulate in the cytoplasm. Since nuclear import of RAD51 is crucial to its function, this study investigated the effect of Cr(VI) on the RAD51 nuclear import mediators, RAD51C and BRCA2. We show acute (24 h) Cr(VI) exposure induces the proper localization of RAD51C and BRCA2. In contrast, prolonged

(120 h) exposure increased the cytoplasmic localization of both proteins, although RAD51C localization was more severely impaired. These results correlate temporally with the previously reported Cr(VI)-induced RAD51 cytoplasmic accumulation. In addition, we found Cr(VI) does not inhibit interaction between RAD51 and its nuclear import mediators. Altogether, our results suggest prolonged Cr(VI) exposure inhibits the nuclear import of RAD51C, and to a lesser extent, BRCA2, which results in the cytoplasmic accumulation of RAD51. Cr(VI)-induced inhibition of nuclear import may play a key role in its carcinogenic mechanism since the nuclear import of many tumor suppressor proteins and DNA repair proteins is crucial to their function.

Keywords: Nuclear import, Particulate chromium (VI), RAD51C, BRCA2, RAD51 cytoplasmic accumulation

Introduction

Particulate hexavalent chromium (Cr(VI)) is an established human lung carcinogen (IARC, 1990). Epidemiological studies of chromate workers published since 1891 have documented the occurrence of Cr(VI)-induced respiratory cancers (Davies et al., 1991; Newman, 1890; Rosenman and Stanbury, 1996). A wealth of data from animal and cell culture studies further supports the carcinogenic potential of this heavy metal (Balansky et al., 2000; Costa et al., 2010; Levy and Venitt, 1986; Xie et al., 2007). Due to its carcinogenicity and frequent occupational and environmental exposure, particulate Cr(VI) is human health concern (IARC, 1990; OSHA, 2015).

A recent study showed Cr(VI) inhibits the high fidelity DNA repair pathway, homologous recombination (HR) in human lung cells (Browning et al., 2016). While HR repair is active after acute (24 h) Cr(VI) exposure, this repair pathway is inhibited by prolonged (120 h) exposure (Browning et al., 2016). Since HR repair is protective against Cr(VI)-induced chromosome damage (Stackpole et al., 2007), the inhibition of this repair pathway is an important component in the carcinogenetic mechanism of Cr(VI). This is exemplified by the fact that the Cr(VI)-induced inhibition of HR repair correlates with increased structural chromosome damage (Browning et al., 2016; Qin et al., 2014).

We have previously shown prolonged Cr(VI) exposure specifically targets the central HR repair protein, RAD51, by inhibiting protein levels and its proper localization to the nucleus (Browning et al., 2016; Qin et al., 2014). Like many DNA repair proteins and tumor suppressors, RAD51 nuclear transport is crucial to its function (Essers et al., 2002; Fabbro and Henderson, 2003; Gildemeister et al., 2009). Nuclear RAD51 levels are tightly regulated to prevent random recombination events from occurring. Upon DNA damage, RAD51 is transported into the nucleus (Essers et al., 2002; Gildemeister et al., 2009). Thus, Cr(VI)-induced RAD51 subcellular mislocalization is an important component of the mechanism of Cr(VI)-induced HR repair inhibition.

Previous studies show BRCA2 and RAD51C both import RAD51 into the nucleus independently of each other

(Gildemeister et al., 2009; Jeyasekharan et al., 2013). We previously observed Cr(VI)-induced RAD51 mislocalization to the cytoplasm corresponded temporally with decreased RAD51C foci formation (Browning et al., 2016). This outcome suggests impaired RAD51 nuclear import may be a factor, for Cr(VI)-induced RAD51 cytoplasmic accumulation. Thus, the goal of this study is to investigate the effect of prolonged Cr(VI) exposure on the RAD51 nuclear import mediator proteins, RAD51C and BRCA2, focusing on their interaction with RAD51 and their subcellular localization.

Materials and Methods

Chemicals and reagents

DMEM and Ham's F12 50:50 mixture and GlutaGRO (L-alanyl-L- glutamine solution) were purchaed from Mediatech Inc (Herndon, VA). Cosmic Calf Serum was purchased from Hyclone (Logan, UT). Dulbecco's phosphate buffered saline (PBS), goat serum, HEPES, penicillin/streptomycin, sodium pyruvate, trypsin/EDTA, Prolong Gold Antifade Reagent with DAPI and Alexa Fluor 488 were purchased from Life Technologies (Grand Island, NY). Plasticware was purchased from BD Biosciences (Franklin Lakes, NJ). Nunc Lab Tek II glass and permanox chamber slides and attachment factor protein were purchased from Thermo Fisher Scientific Inc. (Waltham, MA). FNC coating mix was purchased from AthenaFS (Baltimore, MD). Zinc chromate (CAS#13530-65-9) was purchased from Alfa Aesar (A18178, Ward Hill, MA) and Pfaltz and Bauer (Z00277, Waterbury, CT). 4% paraformaldehyde in PBS was purchased by Alfa Aesar (Ward Hill, MA). Triton X-100, Igepal and Duolink *in situ* orange starter kit mouse/rabbit (DUO92102) were purchased from Sigma-Aldrich (St. Louis, MO). Sodium chloride, 2-mercaptoethanol and bovine serum albumin were purchased from EMD Millipore (Billerica, MA). Potassium chloride, sodium dodecyl sulfate and methanol were purchased from VWR (Randor, PA). Cleland Reagent (DTT), PMSF, Tris, glycine and Tween-20 were purchased from Amresco LLC (Solon, OH). PhosSTOP phosphatase inhibitor tablets and complete ULTRA protease inhibitor tablets were purchesed from Roche Diagnostics Corporation (Indianapolis, IN). PVDF membrane and Bradford assay kit were purchased from BioRad (Hercules, CA) and Odyssey blocking buffer was purchased from LiCor (Lincoln, NE).

Cell culture

We used WTHBF-6 cells, an hTERT immortalized clonal cell line derived from human bronchial fibroblasts, to investigate the effect of Cr(VI) on DNA repair proteins. This cell line has normal growth parameters, a normal stable karyotype and a cytotoxic and clastogenic response to metals similar to primary cells (Wise et al., 2004). To study the RAD51C gene, we also used the model Chinese hamster lung fibroblast cell lines: V79, irs3 and irs3#6. The irs3 cell line, derived from the parental V79 cells, expresses a mutated RAD51C gene resulting in undetectable levels of RAD51C protein. The RAD51C cDNA-complimented cell line, irs3#6 cells, express RAD51C protein levels comparable to the parental cell line, V79. Details and validation of these cells have been previously published (French et

al., 2002; Stackpole et al., 2007). All cells were cultured as adherent monolayers in DMEM/F12 50:50 mixture, supplemented with 15% cosmic calf serum, 1% L-alanyl-L-glutamine, 1% penicillin/streptomycin, and 0.1 mM sodium pyruvate. Cells were maintained in a 5% CO_2-humidified environment at 37°C.

Treatment with particulate Cr(VI) compound

Zinc chromate was administered as a suspension of particles in cold, sterile water as previously described (Xie et al., 2009). After seeding, cells were allowed to reenter logarithmic growth before treatment. Cells were treated for 24, 72 and 120 h with concentrations of 0.1–0.3 µg/cm^2 zinc chromate. Zinc chromate induces a time and concentration dependent increase in cytotoxicity, but plenty of cells survive and proliferate after 120 h exposure to these concentrations (Holmes et al., 2010). We selected 24 h and ≥ 72 h to represent acute and prolonged Cr(VI) exposure, respectively, because RAD51 and HR repair activity differ in human lung cells after 24 and ≥ 72 h exposures to 0.1–0.3 µg/cm^2 zinc chromate (Browning et al., 2016; Qin et al., 2014).

Immunofluorescence

Immunofluorescence staining was conducted as previously described with minor alterations (Xie et al., 2005). Briefly, WTHBF-6 cells were seeded on glass chamber slides coated with FNC while the Chinese hamster cell lines were seeded on permanox chamber slides coated with attachment factor. After zinc chromate treatment, cells were fixed with 4% paraformaldehyde for 10 mins, permeabilized with 0.2% Triton X-100 for 5 mins and blocked with 10% goat serum and 5% BSA in PBS for 1 h. For BRCA2 staining, cells were incubated with 0.5% Triton X-100 for 5 mins, fixed with 4% paraformaldehyde and 0.5% Triton X-100 for 15 mins and blocked with 2.5% BSA. Cells were then incubated with anti-RAD51C (abcam ab72063; 1:500) or anti-BRCA2 (Genetex GTX70121; 1:50) antibodies at 4°C overnight, washed with PBS and incubated with Alexa Fluor 488 (1:3000) for 1 h. Cells were washed with PBS and coverslipped with DAPI. Images of 50 cells per concentration/timepoint were obtained by confocal microscopy. Whole cell and nuclear intensities were measured as integrated intensity using Image J (http://imagej.nih.gov/ij/). Cytoplasmic integrated intensity was determined by subtracting the nuclear intensity value from the whole cell intensity value. Cells with a cytoplasmic intensity greater than the mean intensity + 1 SEM (standard error of the mean) of control cells were considered positive for cytoplasmic accumulation.

Proximity Ligation Assay (PLA)

Cells were seeded in 8 well permanox slides, allowed to reenter logarithmic growth and treated with zinc chromate. After treatment, cells were fixed with 4% paraformaldehyde with 0.5% Triton X for 15 min at 4°C and permeabilized with 0.2% Triton X-100 for 5 min. RAD51/RAD51C and RAD51/BRCA2 wells were blocked for 1 h with 10% goat serum and 5% BSA in PBS or 2.5% BSA in PBS, respectively. RAD51/RAD51C wells were incubated with anti-

RAD51 (GeneTex GTX70230; 1:100) and anti-RAD51C (abcam ab72063; 1:500) while RAD51/BRCA2 wells were incubated with anti-RAD51 (Santa Cruz sc-8349; 1:200) and anti-BRCA2 (Genetex GTX70121; 1:50) at 4°C overnight.

To prepare for the PLA reaction, slides were washed with 1× PBS and the chamber removed, leaving behind the silicon seal. The cells underwent consecutive incubations with; the PLA probes (diluted 1:5) for 1 h; ligation stock and ligase (diluted 1:5 and 1:40) for 30 min; amplification stock and polymerase (diluted 1:5 and 1:80) for 2 h. Slides were washed in 1× Duolink wash buffer A between incubations and all incubations were conducted at 37°C in a humidity chamber. After the last incubation, slides were washed in 1× Duolink wash buffer B, dried, coverslipped with Duolink DAPI and stored at −20°C. A negative control, containing no primary antibodies was used to demonstrate that no background foci formed as a result of the PLA components.

For each protein combination, images of 50 cells per concentration/timepoint were obtained by confocal microscopy. Nikon Elements AR software was used to count the number of foci in each cell and nuclei. The number of cytoplasmic foci was determined by subtracting the number of nuclear foci from the number of whole cell foci.

Western blot

Cells were plated into 100 mm dishes and treated with zinc chromate. After treatment, nuclear extracts were prepared according to our published methods (Qin et al., 2014). Protein concentration was determined with a Bradford assay and 10 μg protein were resolved on 12% Bis-Tris SDS-PAGE gels and transferred to PVDF membranes. Immunoblots were probed with anti-Rad51C (abcam ab55728; 1:250). Equal loading was confirmed by H3 (Cell Signaling 9715; 1:500). Immunoblots of whole cell protein were incubated with Alexa680 or Alexa800 (1:15,000) secondary antibodies and fluorescence detected using an Odyssey Imager (LiCor, Lincon, NE).

Statistics

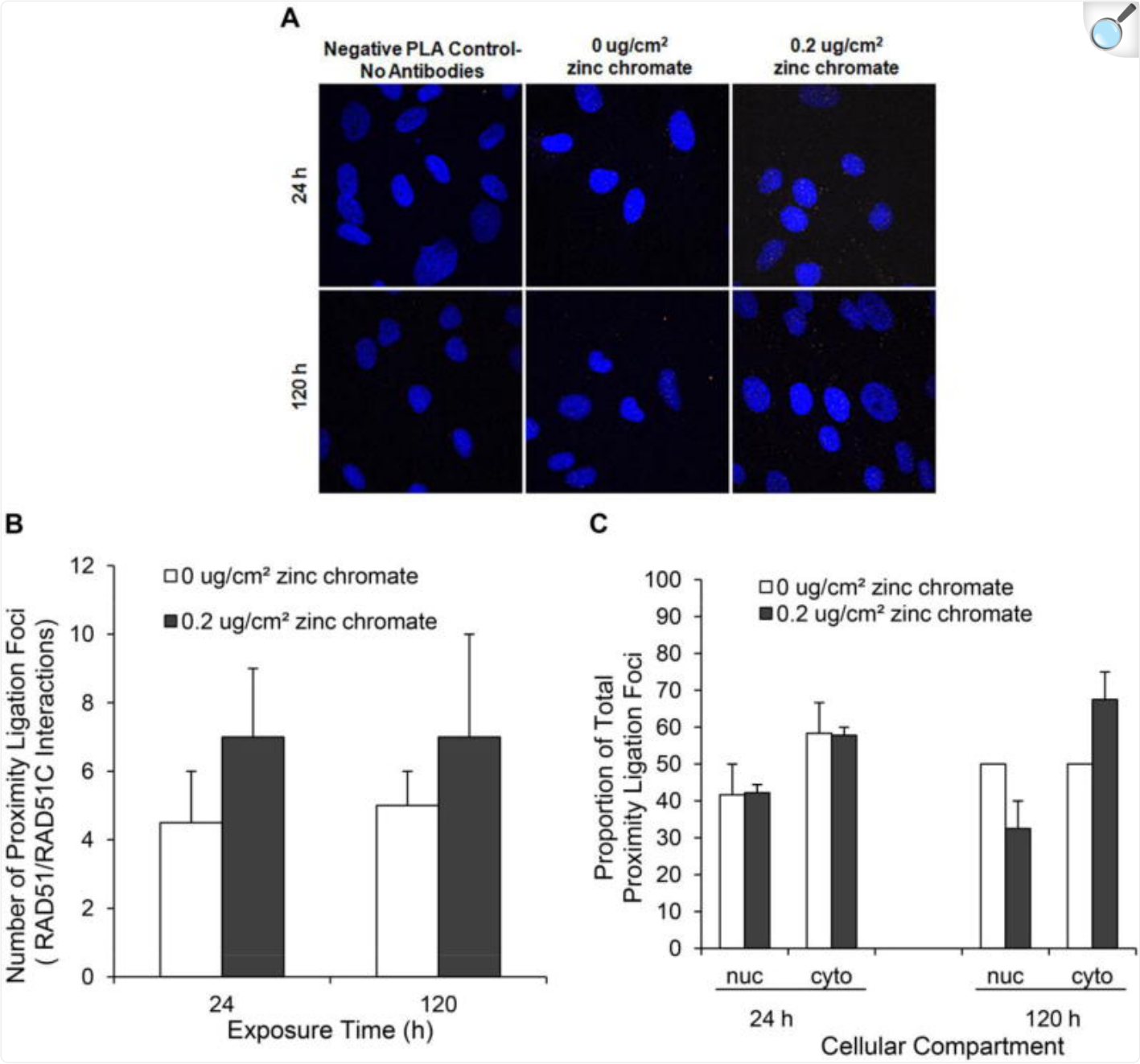
Results are expressed as the mean +/− SEM (standard error of the mean) of three independent experiments. Two-way ANOVA was used to determine the effect of exposure time and Cr(VI) concentration. Tukey's post-hoc analyses waere performed to identify differences between individual means while correcting for multiple comparisons. A 95% confidence interval was constructed for the difference in means of each pair of concentrations. The criterion for statistical significance was $p < 0.05$. All analyses were conducted using GraphPad.

Results

Cr(VI) exposure does not inhibit interactions between RAD51 and RAD51C

We have previously shown RAD51C function, represented by RAD51C foci formation, is active after acute Cr(VI) exposure, but inhibited by prolonged exposure (Browning et al., 2016). Since the inhibition of RAD51C function corresponded temporally with an increase in RAD51 cytoplasmic accumulation, we first investigated the effect of Cr(VI) on RAD51C. We determined the effect of acute (24 h) and prolonged (120 h) Cr(VI) exposure on the interaction between RAD51 and RAD51C by proximity ligation assay. Cr(VI) caused a small increase in RAD51/RAD51C interactions after both 24 and 120 h exposure (Fig. 1A and 1B). For example, 24 and 120 h exposure to 0 and 0.2 ug/cm^2 zinc chromate increased the average number of RAD51/RAD51C interactions per cell from 5 to 7, respectively. Interestingly, Cr(VI) appeared to alter the subcellular distribution of RAD51/RAD51C interactions (Fig. 1C). After 24 h exposure to 0.2 ug/cm^2 zinc chromate, the percent of interactions in cytoplasm was similar to the control level of 58%. Prolonged exposure of 120 h to the same concentration increased the percent of cytoplasmic RAD51/RAD51C interactions from 50 to 68 percent.

Fig 1. Prolonged Cr(VI) exposure does not inhibit RAD51C and RAD51 interaction.



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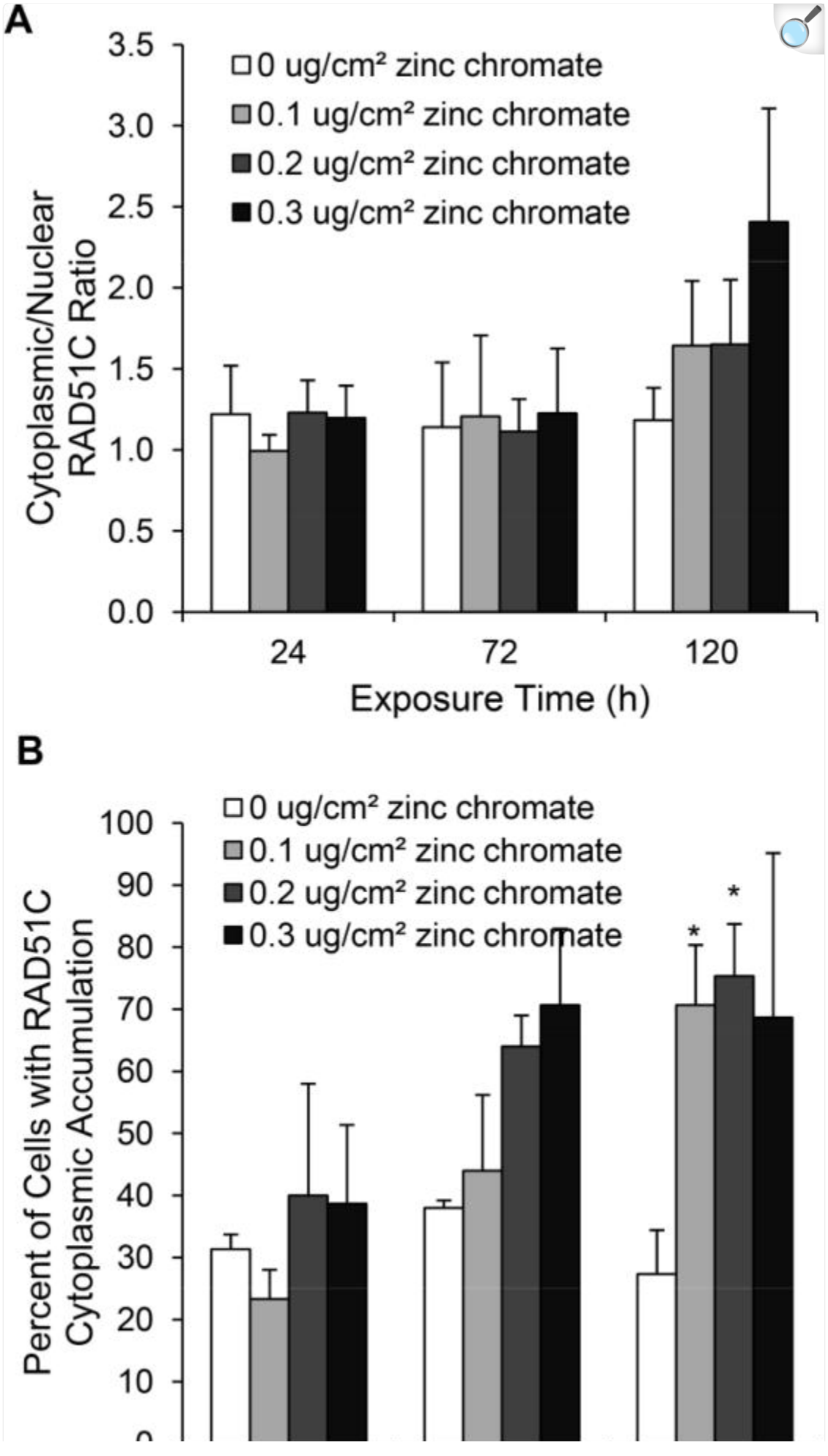
This figure shows prolonged zinc chromate exposure did not reduce the number of proximity ligation foci representing interactions between RAD51C and RAD51, but increased the cytoplasmic proportion of these foci. Data represent an average of two experiments. Error bars = standard error of the mean. **(A)** Representative images of proximity ligation foci representing interactions between RAD51C and RAD51. **(B)** Number of proximity ligation foci per cell. The number of proximity ligation foci increased after 24 and 120 h

exposure to 0.2 ug/cm^2 zinc chromate (not statistically significant). **(C)** Proportion of total proximity ligation foci. The cytoplasmic proportion of proximity ligation foci increased after 120 h zinc chromate exposure (not statistically significant).

Prolonged Cr(VI) exposure induces the subcellular mislocalization of RAD51C

Due to our observed increase in cytoplasmic RAD51/RAD51C interactions, we then examined the effect of Cr(VI) on RAD51C subcellular localization. We found Cr(VI) did not alter the cytoplasmic/nuclear RAD51C ratio after 24 or 72 h exposure (Fig. 2A). However, prolonged Cr(VI) exposure of 120 h increased the cytoplasmic/nuclear ratio. For example, the cytoplasmic/nuclear ratio increased from a control level of 1.2 to 1.6, 1.7 and 2.4 after 120 h exposure to 0.1, 0.2 and 0.3 ug/cm^2 zinc chromate, respectively.

Fig 2. Prolonged Cr(VI) exposure induces the subcellular mislocalization of RAD51C.


A
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0 ug/cm² zinc chromate
0.1 ug/cm² zinc chromate
0.2 ug/cm² zinc chromate
0.3 ug/cm² zinc chromate
Cytoplasmic/Nuclear RAD51C Ratio
24
72
120
Exposure Time (h)
B
100
90
80
70
60
50
40
30
20
10
0 ug/cm² zinc chromate
0.1 ug/cm² zinc chromate
0.2 ug/cm² zinc chromate
0.3 ug/cm² zinc chromate
*
*
Percent of Cells with RAD51C Cytoplasmic Accumulation

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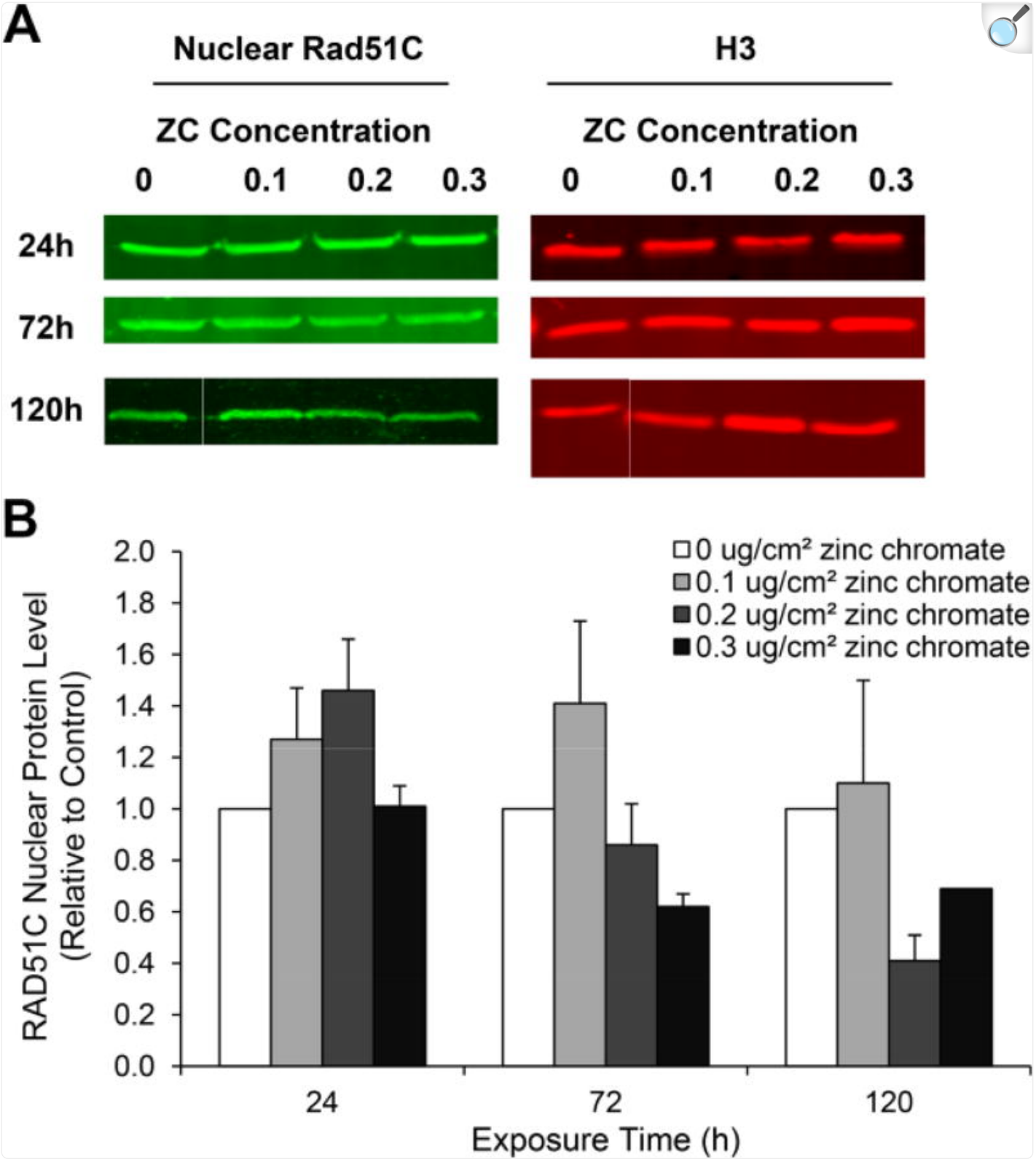
This figure shows prolonged exposure to zinc chromate increased cytoplasmic RAD51C localization. Data represent an average of three experiments. Error bars = standard error of the mean. **(A)** RAD51C cytoplasmic to nuclear ratio determined by fluorescent intensity. The cytoplasmic/nuclear ratio increased after 120 h exposure (not statistically significant). **(B)** Percent of cells with RAD51C cytoplasmic accumulation. Cells with a cytoplasmic intensity greater than the mean + 1 SEM of control cells were considered positive for cytoplasmic accumulation. The percent of cells with RAD51C cytoplasmic accumulation increased after 72 and 120 h exposure and was significantly higher than controls after 120 h exposure to 0.2 ug/cm^2 zinc chromate (*$p<0.05$).

We then calculated the percentage of cells with RAD51C cytoplasmic accumulation, as measured by immunofluorescence intensity, after Cr(VI) exposure. As expected, the data show Cr(VI) did not increase the percent of cells with RAD51C cytoplasmic accumulation after 24 h (Fig. 2B). This corresponded with an increase in nuclear RAD51C protein (Fig. 3A and 3B). However, starting at 72h of exposure, Cr(VI) induced a significant time- and concentration-dependent increase in RAD51C cytoplasmic accumulation ($p=0.0103$ and $p=0.0284$, respectively) (Fig. 2B). For example, 72 h exposure to 0.1, 0.2 and 0.3 ug/cm^2 zinc chromate increased the percent of cells with RAD51 cytoplasmic accumulation from 38 in the control to 44, 68 and 71 percent, respectively. The percent of cells with RAD51C cytoplasmic accumulation increased further after 120 h, increasing from 27 percent in the control to 71, 75 and 69 percent after exposure to 0.1, 0.2 and 0.3 ug/cm^2 zinc chromate, respectively. The observed increase in RAD51C cytoplasmic accumulation corresponded with decreased nuclear RAD51 protein levels (Fig. 3A and 3B). For example, 72 h exposure to 0.1, 0.2 and 0.3 ug/cm^2 zinc chromate produced nuclear RAD51C levels 1.4, 0.86 and 0.62, respectively, relative to controls.

Fig 3. Prolonged Cr(VI) inhibits nuclear RAD51C protein levels.



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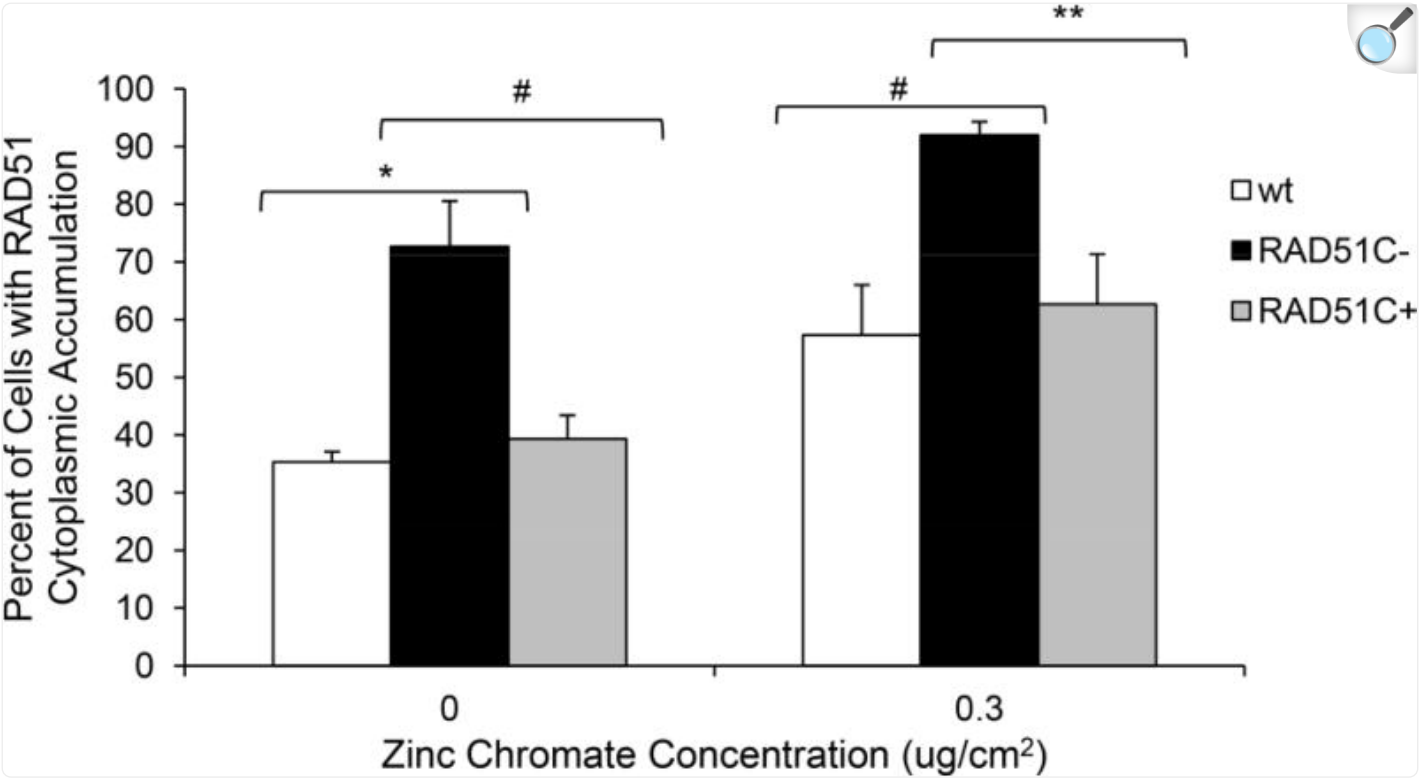
This figure shows zinc chromate exposure increased nuclear RAD51C protein after 24 h, but inhibited nuclear levels after 72 and 120 h. Data represent an average of two experiments. Error bars = standard error of the mean. **(A)** Representative images of nuclear RAD51C western blots. H3 was used as a loading control. **(B)** RAD51C nuclear protein level (relative to control). Nuclear protein levels decreased to lower than control level after 72 and 120h exposure to 0.2 and 0.3 ug/cm^2 zinc chromate (not statistically significant).

RAD51C deficiency induces RAD51 cytoplasmic accumulation

Having determined prolonged Cr(VI) exposure misregulates RAD51C subcellular localization, resulting in a RAD51C deficiency in the nucleus, we then asked whether a RAD51C deficiency can induce the cytoplasmic accumulation of RAD51. To address this question we quantified the percent of cells with RAD51 cytoplasmic accumulation in untreated wild-type, RAD51C deficient (RAD51C−) and RAD51C reconstituted (RAD51C+) Chinese hamster lung cells (Fig. 4). The percent of cells with RAD51 cytoplasmic accumulation was comparable in wild-type and RAD51C reconstituted cells, at 35 and 39 percent, respectively. However, the RAD51C deficient cells exhibited a significantly higher percentage of cells with RAD51 cytoplasmic accumulation ($p=0.0012$). Specifically, 73 percent of RAD51C deficient cells showed RAD51 cytoplasmic accumulation.

Fig 4. RAD51C deficiency is not completely responsible for Cr(VI)-Induced RAD51 cytoplasmic accumulation.



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This figure shows the percent of cells with RAD51 cytoplasmic accumulation was significantly higher in RAD51C deficient cells than wild-type or RAD51C reconstituted cells before and after zinc chromate exposure. The percent of cells with RAD51 cytoplasmic accumulation increased in RAD51C deficient cells after 72 h zinc chromate exposure (not statistically significant). Data represent an average of three experiments. Error bars = standard error of the mean. Statistically different: *$p<0.05$; #$p<0.01$; **$p<0.005$.

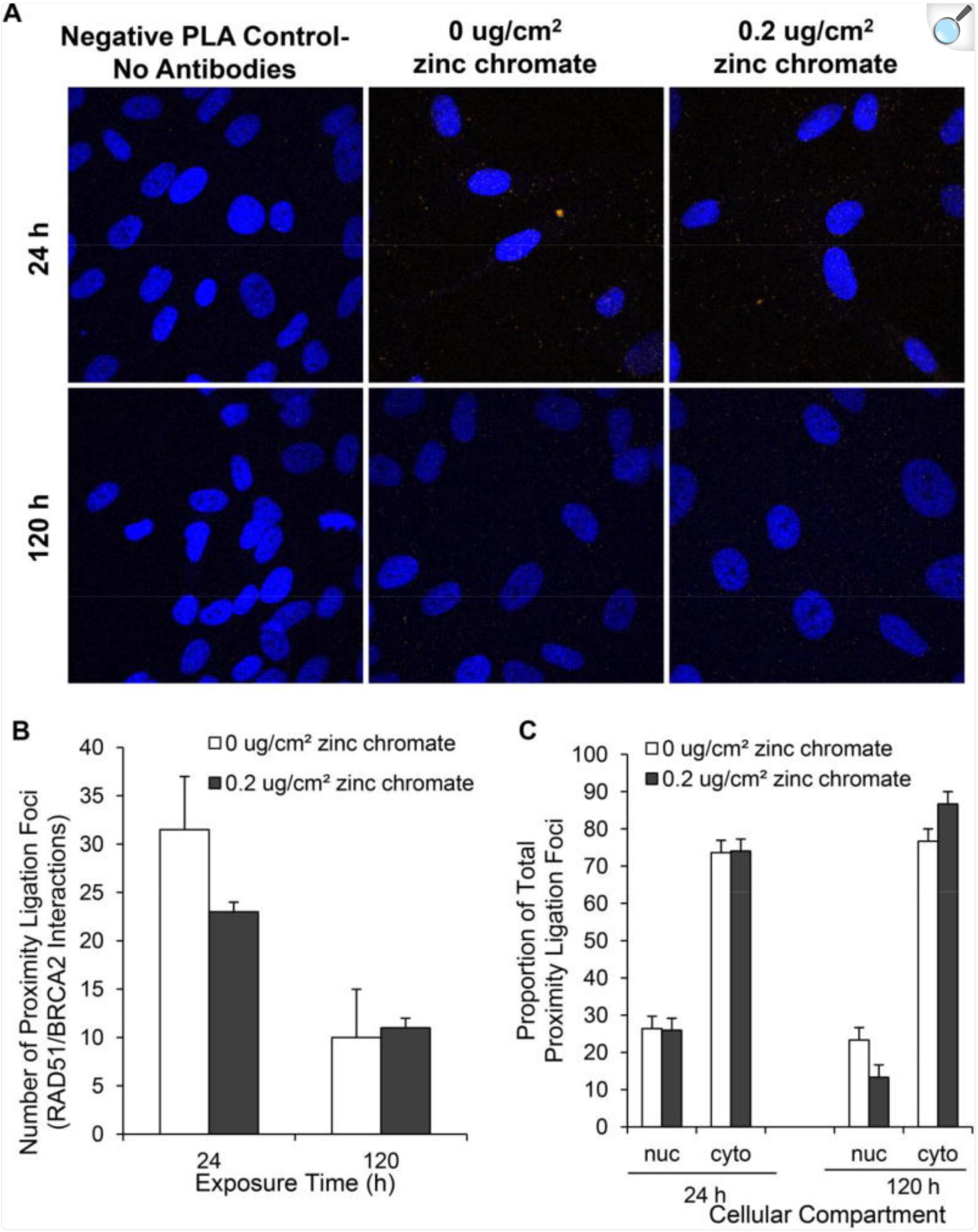
To determine whether a RAD51C deficiency was entirely responsible of the Cr(VI)-induced RAD51 cytoplasmic accumulation, we treated each cell line with zinc chromate for 72 h. If the Cr(VI)-induced inhibition of RAD51C is entirely responsible for the RAD51 cytoplasmic accumulation, we would expect to see no difference in the percent of cells with RAD51 cytoplasmic accumulation between untreated and Cr(VI)-treated RAD51C deficient cells. However, prolonged Cr(VI) exposure induced an increase in the percent of cells with RAD51 cytoplasmic accumulation in the RAD51C deficient cell line (Fig. 4). Specifically, the percent of RAD51C deficient cells with RAD51 cytoplasmic accumulation increased from 73 to 92 percent after zinc chromate exposure. These data suggest a second mechanism

contributes to Cr(VI)-induced RAD51 cytoplasmic accumulation.

Cr(VI) does not inhibit the interaction between RAD51 and BRCA2

We then considered whether the other RAD51 nuclear import mediator, BRCA2, is misregulated by Cr(VI). Although previously published data indicate BRCA2 is functional at the site of the DNA double strand break (Browning et al., 2016), this outcome does not necessarily indicate its role as a RAD51 nuclear import partner is functional. Thus, we began by determining the effect of Cr(VI) on the interaction between RAD51 and BRCA2 using a proximity ligation assay. Interestingly, 24 h Cr(VI) exposure decreased the number of RAD51/BRCA2 interactions, although not significantly (Fig. 5A and 5B). Specifically, 24 h exposure to 0 and 0.2 ug/cm^2 zinc chromate decreased the number of RAD51/BRCA2 interactions per cell from 32 to 23, respectively. In contrast, 120 h Cr(VI) exposure did not inhibit RAD51/BRCA2 interactions. The number of RAD51/BRCA2 interactions per cell remained relatively consistent, showing 10 and 11 PLA foci per cell after exposure to 0 and 0.2 ug/cm^2 zinc chromate, respectively. However, prolonged Cr(VI) exposure appeared to alter the subcellular distribution of RAD51/BRCA2 interactions (Fig. 5C) in a manner similar to that seen with RAD51/RAD51C interactions. After 24 h exposure to 0.2 ug/cm^2 zinc chromate, the percent of interactions in the cytoplasm was similar to the control level of 74 percent. Prolonged exposure of 120 h to the same concentration slightly increased the percent of cytoplasmic RAD51/RAD51C interactions from 77 to 87 percent.

Fig 5. Prolonged Cr(VI) exposure does not inhibit BRCA2 and RAD51 interaction.



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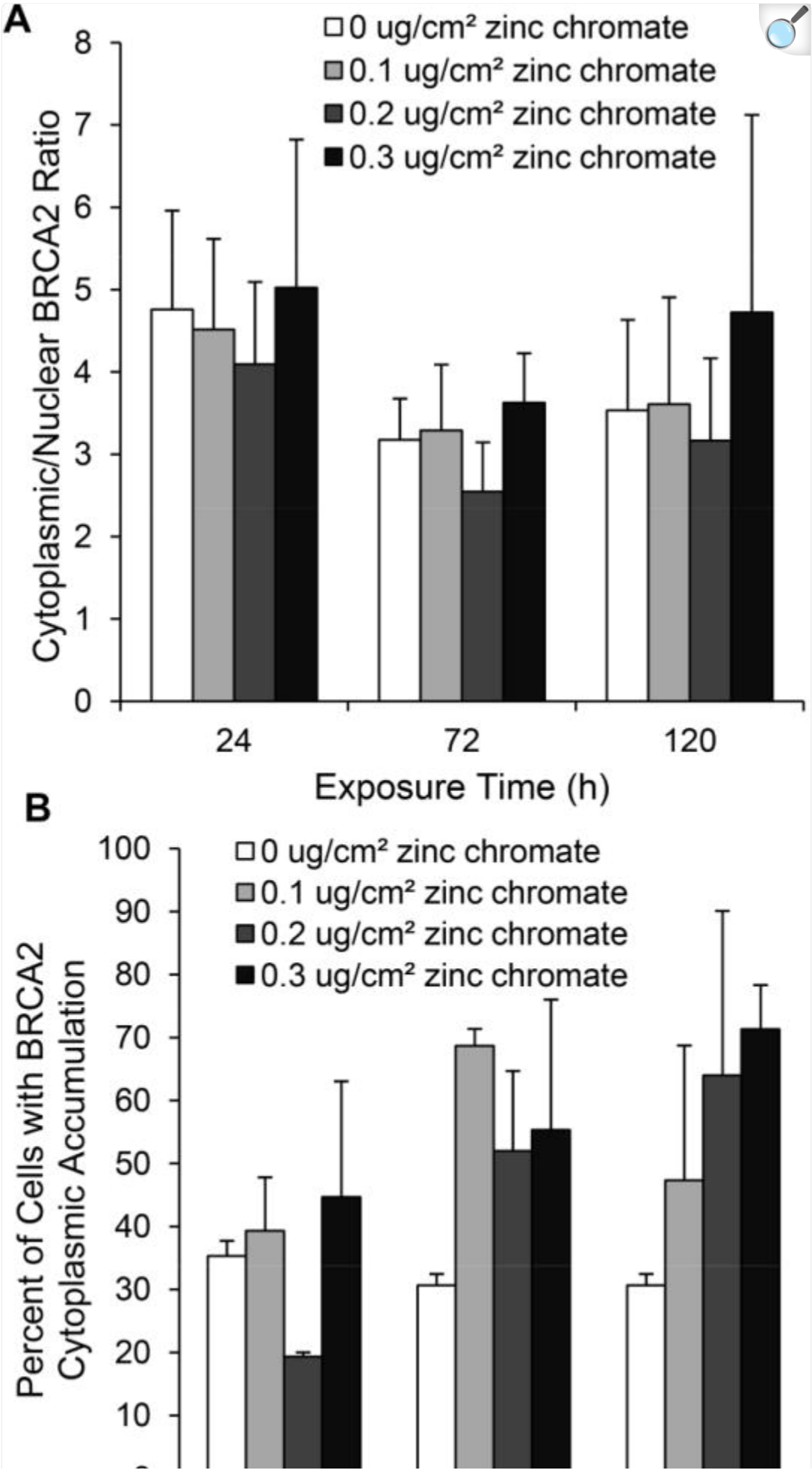
This figure shows prolonged zinc chromate exposure did not reduce the number of proximity ligation foci representing BRCA2 and RAD51 interactions, but increased the cytoplasmic proportion of these foci. Data represent an average of two experiments. Error bars = standard error of the mean. **(A)** Representative images of proximity ligation foci formation representing interactions between BRCA2 and RAD51. **(B)** Number of proximity ligation foci per cell. The number of proximity ligation foci decreased after 24 h exposure to 0.2 ug/cm^2 zinc chromate (not statistically significant), but did not change after 120 h exposure. **(C)** Proportion of total proximity ligation foci. The cytoplasmic proportion of proximity ligation foci increased after 120 h zinc chromate exposure (not statistically significant).

Prolonged Cr(VI) exposure induces a slight increase in BRCA2 subcellular mislocalization

Due to the small observed increase in cytoplasmic RAD51/BRCA2 interactions, we then investigated whether Cr(VI) induces the subcellular mislocalization of BRCA2. The data show Cr(VI) did not increase the cytoplasmic/nuclear BRCA2 ratio after 24 and 72 h exposure (Fig. 6A). Prolonged Cr(VI) exposure of 120 h slightly increased the cytoplasmic/nuclear BRCA2 ratio, but this increase was not significant. Specifically, the cytoplasmic/nuclear BRCA2 ratio increased from 3.5 to 4.7 after 120 h exposure to 0 and 0.3 ug/cm^2 zinc chromate, respectively.

Fig 6. Prolonged Cr(VI) exposure induces the subcellular mislocalization of BRCA2.


A
0 ug/cm² zinc chromate
0.1 ug/cm² zinc chromate
0.2 ug/cm² zinc chromate
0.3 ug/cm² zinc chromate
Cytoplasmic/Nuclear BRCA2 Ratio
0
1
2
3
4
5
6
7
8
24
72
120
Exposure Time (h)
B
0 ug/cm² zinc chromate
0.1 ug/cm² zinc chromate
0.2 ug/cm² zinc chromate
0.3 ug/cm² zinc chromate
Percent of Cells with BRCA2 Cytoplasmic Accumulation
10
20
30
40
50
60
70
80
90
100

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This figure shows prolonged exposure to zinc chromate increased cytoplasmic BRCA2 localization. Data represent an average of three experiments. Error bars = standard error of the mean. **(A)** BRCA2 cytoplasmic to nuclear ratio determined by fluorescent intensity. The cytoplasmic/nuclear ratio varied little at 24 and 72 h exposure, but increased after 120 h exposure to 0.3 ug/cm^2 zinc chromate (not statistically significant). **(B)** Percent of cells with cytoplasmic accumulation of BRCA2. The percent of cells with cytoplasmic accumulation of BRCA2 increased after 72 and 120 h exposure to zinc chromate (not statistically significant).

We then evaluated cytoplasmic accumulation of BRCA2 in individual cells. As expected, Cr(VI) did not significantly increase the percent of cells with BRCA2 cytoplasmic accumulation after 24 h exposure (Fig. 6B). However, 72 and 120 h Cr(VI) exposure increased the percent of cells with BRCA2 cytoplasmic accumulation, although this effect was not significant. For example, 120 h exposure to 0.1, 0.2 and 0.3 ug/cm^2 zinc chromate increased the percent of cells with BRCA2 cytoplasmic accumulation from control levels of 31 percent to 47, 64 and 71 percent, respectively.

Discussion

The inhibition of the high fidelity DNA repair pathway, homologous recombination, allows for the development of Cr(VI)-induced structural chromosome instability (Browning et al., 2016; Qin et al., 2014; Stackpole et al., 2007). RAD51 nuclear localization is crucial to its function during homologous recombination repair (Essers et al., 2002; Gildemeister et al., 2009). While prolonged Cr(VI) exposure has been shown to inhibit RAD51 by inducing its cytoplasmic accumulation, the mechanism of this subcellular mislocalization remains unknown.

This study revealed Cr(VI) inhibits RAD51 nuclear import regulation. Prolonged Cr(VI) exposure induced the subcellular mislocalization of both RAD51 nuclear import mediator proteins, RAD51C and BRCA2. The cytoplasmic accumulation of RAD51C and BRCA2 corresponds temporally with previously reported Cr(VI)-induced RAD51 cytoplasmic accumulation (Browning et al., 2016). While no cytoplasmic increase in RAD51, RAD51C or BRCA2 was observed after acute (24 h) particulate Cr(VI) exposure, a concentration-dependent increase in the percent of cells with cytoplasmic accumulation was observed for each protein after prolonged (120 h) exposures.

Interestingly, we observed the subcellular localization of RAD51C was more adversely affected by prolonged Cr(VI) exposure than BRCA2. The cytoplasmic to nuclear ratio of RAD51C increased in a concentration dependent manner after prolonged Cr(VI) exposure, while that of BRCA2 only increased slightly after exposure to the highest Cr(VI)

concentration. These observations are consistent with our previous report of RAD51C functional inhibition in response to prolonged Cr(VI), while BRCA2 function was not affected (Browning et al., 2016). Together these data indicate that particulate Cr(VI) is less efficient in its inhibition of BRCA2.

We then demonstrated that RAD51C deficiency alone can induce RAD51 cytoplasmic accumulation in a majority of cells. This indicates that the observed Cr(VI)-induced RAD51C inhibition is responsible for the subcellular mislocalization of RAD51. However, the percent of RAD51C-deficient cells with RAD51 cytoplasmic accumulation increased even further (to 92%) after prolonged Cr(VI) exposure. This suggests another factor also contributes to the formation of this phenotype. Previous studies have demonstrated a direct relationship between the cytoplasmic accumulation of BRCA2 and that of RAD51 (Davies et al., 2001; Spain et al., 1999). With this in consideration, we propose that the low level of Cr(VI)-induced mislocalization of BRCA2 may contribute some to the cytoplasmic accumulation of RAD51.

One potential explanation for the lack of nuclear import of RAD51, is that particulate Cr(VI) disrupts the interaction between RAD51 and its import mediators. However, our data do not support this explanation, demonstrating RAD51 interaction with both RAD51C and BRCA2 was not inhibited by prolonged Cr(VI) exposure. The proximity ligation assays also revealed a small, time-dependent shift in the subcellular localization of interactions between RAD51 and both RAD51C and BRCA2. Increases in the cytoplasmic proportion of RAD51/RAD51C and RAD51/BRCA2 interactions were only observed after prolonged Cr(VI) exposure. Although the increases in cytoplasmic localization of these interactions were not significant, alongside our observations of Cr(VI)-induced subcellular mislocalization of RAD51C and BRCA2, these data further support that prolonged Cr(VI) exposure inhibits nuclear import.

A second, albeit untested, explanation for the Cr(VI)-induced inhibition of nuclear import is that Cr ions bind to the nuclear localization signal (NLS). It has previously been shown that Cr can bind to amino acids (Zhitkovich et al., 1996). Specifically, Davis and Scroggie (1974) demonstrated *in vitro* that Cr can bind to arginine, one of the amino acids found at the NLS of both RAD51C and BRCA2 (French et al., 2003; Henderson, 2005). In fact, the classical NLS found in a multitude of proteins contains one or two short sequences of positively charged lysines and arginines (French et al., 2003). Thus, it is possible Cr directly binds to the NLS, blocking its recognition by importins. The binding of Cr to the NLS could also explain why RAD51C subcellular localization is more severely affected than that of BRCA2. RAD51C contains a single NLS, while BRCA2 contains three (French et al., 2003; Henderson, 2005). Accordingly, if Cr inhibits nuclear import by interfering with the NLS, this effect would be more potent to RAD51C than BRCA2.

A final potential explanation for the Cr(VI) induced inhibition of nuclear import is Cr(VI) alters a post-translational modification necessary for nuclear import. While post-translational modifications of the NLS of other proteins directly affects their nuclear import (Christie et al., 2016), no data is available on whether this is true for RAD51C or BRCA2. However, RAD51 has been shown to directly interact with the sumoylation proteins, SUMO-1 and Ubc9, which are both involved in NPC-mediated nuclear transport (Shen et al., 1996; Shima et al., 2013). Saitoh et al. (2002) reported

cytoplasmic localization of Ubc9 coincided with cytoplasmic localization of RAD51 and the inhibition of RAD51 foci formation in the presence of DNA double strand breaks. This suggests Ubc9 may play a role in RAD51 subcellular trafficking.

In summary, our data show prolonged particulate Cr(VI) exposure induces the subcellular mislocalization of RAD51 nuclear import mediators. Our results suggest an inhibition of nuclear import, which could play a pivotal role in the mechanism of Cr(VI)-induced carcinogenesis as the nuclear import of many tumor suppressor proteins and DNA repair protein is crucial to their function. Future research will focus on the mechanism and consequences of impaired nuclear import.

Highlights.

- RAD51C nuclear localization is reduced after prolonged Cr(VI) exposure.
- Prolonged Cr(VI) inhibits nuclear localization of BRCA2 less severely than RAD51C.
- RAD51C deficiency induces the cytoplasmic accumulation of RAD51.
- Cr(VI) does not inhibit interactions between RAD51 and its nuclear import partners.

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Footnotes

Conflict of Interest Statement

There are no other conflicts to declare.

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